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(54) Title: IMMUNOTHERAPY OF CANCER AND INFECTIOUS DISEASE USING ANTIGEN-PRESENTING CELLS SENSITIZED WITH HEAT SHOCK PROTEIN-ANTIGEN COMPLEXES

(57) Abstract

The present invention relates to methods and compositions for enhancing immunological responses and for the prevention and treatment of infectious diseases or primary and metastatic neoplastic diseases based on the administration of macrophages and/or other antigen presenting cells (APC) sensitized with heat shock proteins non-covalently bound to peptide complexes and/or antigenic components. APC are incubated in the presence of hsp-peptide complexes and/or antigenic components in vitro. The sensitized cells are reinfused into the patient with or without treatment with cytokines including but not limited to interferon-α, interferon-α, interleukin-2, interleukin-4, interleukin-6 and tumor neurosis factor.

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"Immunotherapy of Cancer and Infectious Disease Using Antigen-Presenting Cells
Sensitized with Heat Shock Protein-Antigen Complexes"

1. INTRODUCTION

The present invention relates to compositions and
methods of adoptive immunotherapy for the prevention and/or
treatment of primary and metastatic cancer and other
immunological or infectious disorders in humans using
macrophages sensitized with non-covalent complexes of heat
shock protein (hsp) and antigenic molecules. In the practice
of the adoptive immunotherapy as a method of the invention,
compositions of hsp including, but not limited to, hsp70,
hsp90, gp96 alone or in combination with each other, non-
covalently bound to antigenic molecules, are used to
sensitize macrophages in vitro to augment immune responses to
tumors and infectious agents in vivo, upon infusion into the
patient. In the practice of the prevention and treatment
methods against infectious diseases and cancer, complexes of
antigenic molecules and heat shock/stress proteins (hsps)
including, but not limited to, hsp70, hsp90, gp96 alone or in
combination with each other, are used to augment the immune
response to genotoxic and nongenotoxic factors, tumors and
infection.

2. BACKGROUND OF THE INVENTION

Adoptive immunotherapy of cancer refers to a therapeutic
approach in which immune cells with an antitumor reactivity
are administered to a tumor-bearing host, with the aim that
the cells mediate either directly or indirectly, the
regression of an established tumor. Transfusion of
lymphocytes, particularly T lymphocytes, falls into this

category and investigators at the National Cancer Institute
(NCI) have used autologous reinfusion of peripheral blood
lymphocytes or tumor-infiltrating lymphocytes (TIL), T cell
cultures from biopsies of subcutaneous lymph nodules, to
5 treat several human cancers (Rosenberg, S.A., U.S. Patent No.
4,690,914, issued September 1, 1987; Rosenberg, S.A., et al.,
1988, *N. England J. Med.* 319:1676-1680). For example, TIL
expanded *in vitro* in the presence of interleukin (IL)-2 have
been adoptively transferred to cancer patients, resulting in
10 tumor regression in select patients with metastatic melanoma.
Melanoma TIL grown in IL-2 have been identified as activated
T lymphocytes CD3$^+$ HLA-DR$^+$, which are predominantly CD8$^+$
cells with unique *in vitro* antitumor properties. Many long-
term melanoma TIL cultures lyse autologous tumors in a
15 specific MHC class I- and T cell antigen receptor dependent
manner (Topalian, S.L., et al., 1989, *J. Immunol.* 142:3714).
However, studies of TIL derived from other types of tumors
have revealed only scant evidence for cytolytic or
proliferative antitumor immune specificity (Topalian, S.L. et
20 al., 1990, in *Important Advances in Oncology*, V.T. DeVita,
S.A. Hellman and S.A. Rosenberg, eds. J.B. Lippincott,
Philadelphia, pp. 19-41). In addition, the toxicity of the
high-dose IL-2 + activated lymphocyte treatment advocated by
the NCI group has been considerable, including high fevers,
25 severe rigors, hypotension, damage to the endothelial wall
due to capillary leak syndrome, and various adverse cardiac
events such as arrhythmias and myocardial infarction
(Rosenberg S.A., et al., 1988, *N. England J. Med.* 319:1676-
1680).
30

2.1. Tumor-Specific Immunogenicities of Heat Shock/Stress Proteins hsp70, hsp90 and gp96

Srivastava et al. demonstrated immune response to
35 syngeneic or cancer in methylcholanthrene-induced sarcomas of
inbred mice (1988, *Immunol. Today* 9:78-83). In these
studies, it was found that the molecules responsible for the

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individually distinct immunogenicity of these tumors were
identified as cell-surface glycoproteins of 96kDa (gp96) and
intracellular proteins of 84 to 86kDa (Srivastava, P.K., et
al., 1986, *Proc. Natl. Acad. Sci. USA* 83:3407-3411; Ullrich,
5 S.J., et al., 1986, *Proc. Natl. Acad. Sci. USA* 83:3121-3125).
Immunization of mice with gp96 or p84/86 isolated from a
particular tumor rendered the mice immune to that particular
tumor, but not to antigenically distinct tumors. Isolation
and characterization of genes encoding gp96 and p84/86
10 revealed significant homology between them, and showed that
gp96 and p84/86 were, respectively, the endoplasmic reticular
and cytosolic counter parts of the same heat shock proteins
(Srivastava, P.K., et al., 1988, *Immunogenetics* 28:205-207;
Srivastava, P.K., et al., 1991, *Curr. Top. Microbiol.*
15 *Immunol.* 167:109-123). Further, hsp70 was shown to elicit
immunity to the tumor from which it was isolated but not to
antigenically distinct tumors. However, hsp70 depleted of
peptides was found to lose its immunogenic activity (Udono,
M., and Srivastava, P.K., 1993, *J. Exp. Med.* 178:1391-1396).
20 These observations suggested that the heat shock proteins are
not immunogenic *per se*, but are carriers of antigenic
peptides that elicit specific immunity to cancers
(Srivastava, P.K., 1993, *Adv. Cancer Res.* 62:153-177).

25   2.2. <u>Cells Involved in Immune Responses</u>
     Cells of the immune system arise from pluripotent stem
cells through two main lines of differentiation: a) the
lymphoid lineage producing lymphocytes (T cells, B cells
natural killer cells), and b) the myeloid lineage producing
30 phagocytes (monocytes, macrophages and neutrophils) and other
accessory cells (antigen-presenting cells, platelets, mast
cells and endothelial cells) (Roitt I, Brostoff J. and Male
D., (eds.), 1993, *Immunology*, Ch. 11, Mosby London).
     In the myeloid lineage, the pluripotent stem cells
35 generate colony-forming units (CFU) which can give rise to
granulocytes, erythrocytes, monocytes and megakaryocytes
(CFU-GEMM). Interleukin-3 and granulocyte-macrophage-colony

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simulating factor (GM-CSF) are required for further
maturation of granulocytes and monocytes. Thus monocytes
present in peripheral blood leukocytes can be activated with
GM-CSF *in vitro* and are capable of becoming macrophages
5 (Gradstein, K.H., et al., 1986, *Science* 232:506-508). Other
cytokines, e.g., IL-1, IL-4 and IL-6 promote the
differentiation of monocytes into macrophages (Clark S.C. and
Kamen R., 1987, *Science* 236:1229-1237).

Thus, macrophages originate in the bone marrow from
10 myeloid stem cells. They circulate in the blood as monocytes
and undergo final differentiation to mature tissue
macrophages in liver, spleen and lung, among other tissues.
Macrophages appear to be major effector cells and leukocytes
such as T-lymphocytes and NK cells also play a part in cell-
15 mediated cytotoxicity.

3. SUMMARY OF THE INVENTION

The present invention provides compositions comprising
macrophages and/or other antigen presenting cells (APC)
20 sensitized with complexes of heat shock proteins (hsps) non-
covalently bound to antigenic molecules, and methods
comprising administering such compositions in
pharmaceutically acceptable carriers to human subjects with
cancer or infectious diseases. The preferred hsps comprised
25 in the complexes suitable for sensitizing the macrophages
include, but are not limited to hsp70, hsp90 and gp160 or a
combination thereof. Such cells sensitized by complexes
comprising hsps and antigenic molecules are herein referred
to as "hsp-sensitized" cells.
30   The present invention encompasses methods for adoptive
immunotherapy of cancer and infectious diseases by enhancing
the host's immunocompetence and activity of immune effector
cells. Adoptive immunotherapy with hsp-sensitized
macrophages and other antigen-presenting cells (APC), for
35 example, dendritic cells and B cells (B lymphocytes), induces
specific immunity to tumor cells and/or antigenic components,
promoting regression of the tumor mass or treatment of

immunological disorders or infectious diseases, as the case
may be.

The present invention also encompasses methods for the
prevention of cancer, infectious diseases and immunological
5 disorders using the adoptive immunotherapy methods of the
invention.

In another embodiment, the methods optionally further
comprise administering biological response modifiers, e.g.,
cytokines such as interferon (IFN)-α, IFN-γ, interleukin
10 (IL)-2, IL-4, IL-6, tumor necrosis factor (TNF) or other
cytokine growth factors.

4.   **BRIEF DESCRIPTION OF FIGURES**

Figure 1. PEC sensitized with gp96 preparations derived
15 from N1 cells are recognized by VSV-specific cytotoxic T-
lymphocytes (CTLs), in Kb - restricted, CD8-mediated manner,
but PEC pulsed with gp96 derived from EL4 cells are not. **(A)**
Pristane-induced PEC (1x10⁴) from C57BL/6 mice and VSV peptide
specific CTL (5x10⁴) from C57BL/6 mice and VSV peptide
20 specific CTL (5x10⁴) were co-cultured in presence of gp96 (2
or 10μg/ml) derived from N1 or EL4 cells in 96-well U-bottom
plate at 37°C. After 24 hr supernatants were collected and
TNF-α production was measured by bioassay in a cytotoxicity
assay using WEHI164 cells. WEHI164 cells were seeded
25 (2,500/well) in flat-bottom 96 well plates. Serially diluted
supernatants of PEC-CTL coculture were added. γ-TNF-α was
cultured with WEHI 164 in separate wells as control. After 4
hr culture at 37°C, 50μl MTT (1mg/ml) was added, with a 4 hr
incubation, followed by 100μl propanol-0.05M Hcl. O.D. (590
30 nm) was measured immediately. Sample concentrations were
calculated by comparison with dilution points which resulted
in killing of 50% of WEHI164 cells. **(B)** The ability of
gp96-sensitized macrophage to act as targets in CTL assays
was tested. PEC (5x10⁶/ml) were pulsed with gp96 (10μg/ml)
35 derived from N1 cells (closed circle), or EL4 cells (open
circle), or with VSV nucleocapsid Kᵇ epitope peptide (10 μM)
(closed triangle), as a positive control, or medium control

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(open triangle) for 2 hr at 37°C, followed by labeling with
^{51}Cr for 1.5 h. These cells were used as targets in 4 hr ^{51}Cr-
release assay with VSV-specific CTL.   (C)   Anti-CD4 mAb
(GK1.5) ascites (open circle), anti-CD8 mAb (YTS1694) (closed
5 square), anti-H-2K^b mAb (Y3) (closed circle), anti-H-2D^b mAb
(B22.249) (open triangle) (obtained from American Type
Culture Collection, Washington, D.C.) or RPMI control (*)
were added to the CTL assay at the same time as effector
cells and ^{51}Cr-labelled PEC pulsed with N1 gp96 (E/T ratio =
10 10).

        Figure 2.   Tumor growth, measured as average tumor
diameter in 5 groups of mice challenged with 1x10^5 cells of
Methylcholanthrene (Meth)- A - induced tumor cells treated as
15 follows: A. subcutaneous injection of buffer solution; B.
subcutaneous injection containing 9μg gp96-peptide complexes
derived from liver tissue; C. intraperitoneal injection with
5x10^6 peritoneal exudate cells (PEC) sensitized with 9μg gp96-
peptide complexes derived from normal liver (Liver gp96 +
20 PEC); D. subcutaneous injection containing 9μg gp96-peptide
complexes derived from Meth A tumor cells; and E.
intraperitoneal injection with 5x10^6 PEC sensitized with 9μg
gp96-peptide complexes derived from Meth A tumor cells (Meth
A gp96 + PEC).
25

        5.   DETAILED DESCRIPTION OF THE INVENTION
        The present invention pertains to compositions
comprising antigen presenting cells (APC) sensitized with
complexes of heat shock proteins (hsps) non-covalently bound
30 to antigenic molecules, and therapeutic and prophylactic
methods comprising administering such compositions in
pharmaceutically acceptable carriers to individuals,
preferably human, suffering from cancer, infectious diseases
or immunological disorders. The APC can be selected from
35 among those antigen presenting cells known in the art,
including but not limited to macrophages, dendritic cells,

B lymphocytes, and a combination thereof, and are preferably
macrophages. The preferred hsps comprised in the complexes
suitable for sensitizing the macrophages and/or APC include,
but are not limited to, hsp70, hsp90, gp96 and gp100 or a
5 combination thereof. In a preferred embodiment, the
sensitized cells are human cells. In another preferred
embodiment, the complexes used to sensitize the cells
comprise human hsps and human antigenic molecules. The hsp-
sensitized cells can be autologous or non-autologous (eg.,
10 allogeneic) to the patient.

In the preferred use of cells autologous to the
individual, the use of autologous immune cells such as
lymphocytes, macrophages or other APC in adoptive
immunotherapy, circumvents a major ethical problem - the
15 issue of whom to select as the donor of the immune cells for
adoptive transfer. One cannot immunize normal individuals or
even cancer patients in order to obtain immune cells, because
of the danger that the inactivated antigenic tumor might grow
in the individual who was to be the immunized donor. Another
20 problem circumvented by use of autologous immune cells is
graft versus host disease which can be fatal if
unsuccessfully treated.

Adoptive immunotherapy according to the invention allows
activation of immune cells by incubation with hsp-antigenic
25 molecule complexes, and measurement of reactivity against the
tumor or infectious agent *in vitro*. This *in vitro* boost
followed by clonal selection and/or expansion, and patient
infusion constitutes a useful therapeutic/prophylactic
strategy.

30   "Antigenic molecule" as used herein refers to the
peptides with which the hsps are endogenously associated *in
vivo* (e.g., in infected cells or precancerous or cancerous
tissue) as well as exogenous antigens/immunogens (i.e., with
which the hsps are not complexed *in vivo*) and
35 antigenic/immunogenic fragments and derivatives thereof.

Heat shock proteins, which are also referred to
interchangeably herein as stress proteins, useful in the

practice of the instant invention can be selected from among any cellular protein that satisfies any one of the following criteria. It is a protein whose intracellular concentration increases when a cell is exposed to a stressful stimuli, it

5 is capable of binding other proteins or peptides, and it is capable of releasing the bound proteins or peptides in the presence of adenosine triphosphate (ATP) or low pH, or it is a protein showing at least 35% homology with any cellular protein having any of the above properties.

10   The first stress proteins to be identified were the heat shock proteins (hsps). As their name implies, hsps are synthesized by a cell in response to heat shock. To date, three major families of hsp have been identified based on molecular weight. The families have been called hsp60, hsp70

15 and hsp90 where the numbers reflect the approximate molecular weight of the stress proteins in kilodaltons. Many members of these families were found subsequently to be induced in response to other stressful stimuli including, but not limited to, nutrient deprivation, metabolic disruption,

20 oxygen radicals, and infection with intracellular pathogens. (See Welch, May 1993, *Scientific American* 56-64; Young, 1990, *Annu. Rev. Immunol.* 8:401-420; Craig, 1993, *Science* 260:1902-1903; Gething, et al., 1992, *Nature* 355:33-45; and Lindquist, et al., 1988, *Annu. Rev. Genetics* 22:631-677), the

25 disclosures of which are incorporated herein by reference. It is contemplated that hsps/stress proteins belonging to all of these three families can be used in the practice of the instant invention.

The major hsps can accumulate to very high levels in

30 stressed cells, but they occur at low to moderate levels in cells that have been stressed. For example, the highly inducible mammalian hsp70 is hardly detectable at normal temperatures but becomes one of the most actively synthesized proteins in the cell upon heat shock (Welch, et al., 1985, *J.*

35 *Cell. Biol.* 101:1198-1211). In contrast, hsp90 and hsp60 proteins are abundant at normal temperatures in most, but not all, mammalian cells and are further induced by heat (Lai, et

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al., 1984, *Mol. Cell. Biol.* 4:2802-10; van Bergen en
Henegouwen, et al., 1987, *Genes Dev.* 1:525-31).

Heat shock proteins are among the most highly conserved
proteins in existence. For example, DnaK, the hsp70 from E.
5 coli has about 50% amino acid sequence identity with hsp70
proteins from excoriates (Bardwell, et al., 1984, *Proc. Natl.
Acad. Sci.* 81:848-852). The hsp60 and hsp90 families also
show similarly high levels of intra families conservation
(Hickey, et al., 1989, *Mol. Cell. Biol.* 9:2615-2626; Jindal,
10 1989, *Mol. Cell. Biol.* 9:2279-2283). In addition, it has
been discovered that the hsp60, hsp70 and hsp90 families are
composed of proteins that are related to the stress proteins
in sequence, for example, having greater than 35% amino acid
identity, but whose expression levels are not altered by
15 stress. Therefore it is contemplated that the definition of
stress protein, as used herein, embraces other proteins,
muteins, analogs, and variants thereof having at least 35% to
55%, preferably 55% to 75%, and most preferably 75% to 85%
amino acid identity with members of the three families whose
20 expression levels in a cell are enhanced in response to a
stressful stimulus. The purification of stress proteins
belonging to these three families is described below.

The hsp-antigenic molecule complexes used to sensitize
APCs according to the invention may include any complex
25 containing an hsp and a molecule that is capable of inducing
an immune response in a mammal. The antigenic/immunogenic
molecules are noncovalently associated with the hsp.
Preferred complexes include, but are not limited to, hsp60-
peptide, hsp70-peptide and hsp90-peptide complexes, in which
30 the hsp-peptide complex is present *in vivo* and is isolated
from cells. In a specific embodiment, the complex comprises
an hsp called gp96 which is present in the endoplasmic
reticulum of eukaryotic cells and is related to the
cytoplasmic hsp90's.

35   Although the hsps can be allogeneic to the patient, in a
preferred embodiment, the hsps are autologous to (derived
from) the patient to whom they are administered. The hsps

and/or antigenic molecules can be purified from natural sources, chemically synthesized, or recombinantly produced.

The present invention encompasses methods for adoptive immunotherapy of cancer, infectious diseases and

5 immunological disorders by enhancing the host's immunocompetence activity of immune effector cells. Adoptive immunotherapy with macrophages and other APC for example, dendritic cells and B-cells, sensitized hsps and antigenic molecules, induces specific immunity to tumor cells and/or

10 infectious agents (depending upon the identity of antigenic molecule) and leads to regression of the tumor mass and/or infectious disease, respectively.

Cancers which are responsive to adoptive immunotherapy by the hsp complex-sensitized macrophages and/or APC of the

15 invention include, but are not limited to human sarcomas and carcinomas, e.g., fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's

20 tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas,

25 cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma,

30 epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma; leukemias, e.g., acute lymphocytic leukemia and acute myelocytic leukemia

35 (myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia); chronic leukemia (chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia);

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and polycythemia vera, lymphoma (Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenström's macroglobulinemia, and heavy chain disease.

The invention also provides a kit comprising in a
5 container a complex of an hsp noncovalently bound to an exogenous antigenic molecule. The kit can further comprise in a second container human APC.

5.1 Complexes for Sensitizing APC

10   In accordance with the methods described herein, APC are sensitized with hsp complexed with immunogenic or antigenic molecules that are endogenously complexed to hsps or MHC antigens and can be used as antigenic molecules. For example, such peptides may be prepared that stimulate
15 cytotoxic T cell responses against different tumor specific antigens (e.g., tyrosinase, gp100, melan-A, gp75, mucins, etc.) and viral proteins including, but not limited to, proteins of immunodeficiency virus type I (HIV-I), human immunodeficiency virus type II (HIV-II), hepatitis type A,
20 hepatitis type B, hepatitis type C, influenza, Varicella, adenovirus, herpes simplex type I (HSV-I), herpes simplex type II (HSV-II), rinderpest, rhinovirus, echovirus, rotavirus, respiratory syncytial virus, papilloma virus, papova virus, cytomegalovirus, echinovirus, arbovirus,
25 huntavirus, coxsackie virus, mumps virus, measles virus, rubella virus and polio virus. In the embodiment wherein the antigenic molecules are peptides noncovalently complexed to hsps in vivo, the complexes can be isolated from cells, or alternatively, produced in vitro from purified preparations
30 each of hsps and antigenic molecules.

In another specific embodiment, antigens of cancers (e.g., tumors) or infectious agents (e.g., viral antigen, bacterial antigens, etc.) can be obtained by purification from natural sources, by chemical synthesis, or
35 recombinantly, and, through in vitro procedures such as that described below, noncovalently complexed to hsps.

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In an embodiment wherein the hsp-antigenic molecule complex to be used is a complex that is produced *in vivo* in cells, exemplary purification procedures such as described in Sections 5.1.1-5.1.3 below can be employed. Alternatively, 5 in an embodiment wherein one wishes to use antigenic molecules by complexing to hsps *in vitro*, hsps can be purified for such use from the endogenous hsp-peptide complexes in the presence of ATP or low pH (or chemically synthesized or recombinantly produced). The protocols 10 described herein may be used to isolate hsp-peptide complexes, or the hsps alone, from any eukaryotic cells for example, tissues, isolated cells, or immortalized eukaryote cell lines infected with a preselected intracellular pathogen, tumor cells or tumor cell lines.

15

5.1.1.   Preparation and Purification of Hsp 70-peptide Complexes

The purification of hsp 70-peptide complexes has been described previously, see, for example, Udono et al., 1993, 20 *J. Exp. Med.* 178:1391-1396. A procedure that may be used, presented by way of example but not limitation, is as follows:

Initially, tumor cells are suspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer (pH7), 25 150mM NaCl, 2mM CaCl$_2$, 2mM MgCl$_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. As an alternative to sonication, the cells may be lysed by mechanical shearing and in this approach the 30 cells typically are resuspended in 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 minutes and then homogenized in a dounce homogenizer until >95% cells are lysed.

Then the lysate is centrifuged at 1,000g for 10 minutes 35 to remove unbroken cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at 100,000g for 90 minutes, the supernatant harvested and then mixed with Con A

Sepharose equilibrated with phosphate buffered saline (PBS) containing 2mM Ca^{2+} and 2mM Mg^{2+}. When the cells are lysed by mechanical shearing the supernatant is diluted with an equal volume of 2X Lysis buffer prior to mixing with Con A

5 Sepharose. The supernatant is then allowed to bind to the Con A Sepharose for 2-3 hours at 4°C. The material that fails to bind is harvested and dialyzed for 36 hours (three times, 100 volumes each time) against 10mM Tris-Acetate pH 7.5, 0.1mM EDTA, 10mM NaCl, 1mM PMSF. Then the dialyzate is

10 centrifuged at 17,000 rpm (Sorvall SS34 rotor) for 20 minutes. Then the resulting supernatant is harvested and applied to a Mono Q FPLC column equilibrated in 20mM Tris-Acetate pH 7.5, 20mM NaCl, 0.1mM EDTA and 15mM 2-mercaptoethanol. The column is then developed with a 20mM to

15 500mM NaCl gradient and then eluted fractions fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and characterized by immunoblotting using an appropriate anti-hsp70 antibody (such as from clone N27F3-4, from StressGen).

20   Fractions strongly immunoreactive with the anti-hsp70 antibody are pooled and the hsp70-peptide complexes precipitated with ammonium sulfate; specifically with a 50%-70% ammonium sulfate cut. The resulting precipitate is then harvested by centrifugation at 17,000 rpm (SS34 Sorvall

25 rotor) and washed with 70% ammonium sulfate. The washed precipitate is then solubilized and any residual ammonium sulfate removed by gel filtration on a Sephadex[R] G25 column (Pharmacia). If necessary the hsp70 preparation thus obtained can be repurified through the Mono Q FPCL Column as

30 described above.

    The hsp70-peptide complex can be purified to apparent homogeneity using this method. Typically 1mg of hsp70-peptide complex can be purified from 1g of cells/tissue.

    The present invention further describes a new and rapid
35 method for purification of hsp70-peptide complexes. This improved method uses chromatography with ADP affixed to a solid substratum (e.g., ADP-agarose). The resulting hsp70

preparations are higher in purity and devoid of peptides. The hsp70 yields are also increased significantly by about more than 10 fold. Alternatively, chromatography with nonhydrolyzable analogs of ATP, instead of ADP, can be used
5 in chromatography for purification of hsp70-peptide complexes. By way of example but not limitation, purification of hsp70-peptide complexes by ADP-agarose chromatography was carried out as follows: Meth A sarcoma cells (500 million cells) were homogenized in hypotonic
10 buffer and the lysate was centrifuged at 100,000 g for 90 minutes at 4°C. The supernatant was divided into two and was applied to an ADP-agarose or an ATP-agarose column. The columns were washed in buffer and were eluted with 3 Mm ADP or 3 mM ATP, respectively. The eluted fractions were
15 analyzed by SDS-PAGE: in both cases, apparently homogeneous preparations of hsp70 were obtained. However, when each of the preparations was tested for presence of peptides, the ADP-bound/eluted hsp70 preparation was found to be associated with peptides, while the ATP-bound/eluted hsp70 preparation
20 was not.

5.1.2. Preparation and Purification of Hsp 90-peptide Complexes

A procedure that can be used, presented by way of example and not limitation, is as follows:

25   Initially, tumor cells are suspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer pH 7, 150mM NaCl, 2mM $CaCl_2$, 2mM $MgCl_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic
30 examination. As an alternative to sonication, the cells may be lysed by mechanical shearing and in this approach the cells typically are resuspended in 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 minutes and then homogenized in a dounce homogenizer until >95% cells are
35 lysed.

Then the lysate is centrifuged at 1,000g for 10 minutes to remove unbroken cells, nuclei and other cellular debris.

The resulting supernatant is recentrifuged at 100,000g for
90 minutes, the supernatant harvested and then mixed with Con
A Sepharose equilibrated with PBS containing 2mM Ca^{2+} and 2mM
Mg^{2+}. When the cells are lysed by mechanical shearing the
5 supernatant is diluted with an equal volume of 2X Lysis
buffer prior to mixing with Con A Sepharose. The supernatant
is then allowed to bind to the Con A Sepharose for 2-3 hours
at 4°C. The material that fails to bind is harvested and
dialyzed for 36 hours (three times, 100 volumes each time)
10 against 10mM Tris-Acetate pH 7.5, 0.1mM EDTA, 10mM NaCl, 1mM
PMSF. Then the dialyzate is centrifuged at 17,000 rpm
(Sorvall SS34 rotor) for 20 minutes. Then the resulting
supernatant is harvested and applied to a Mono Q FPLC column
equilibrated with lysis buffer. The proteins are then eluted
15 with a salt gradient of 200mM to 600mM NaCl.

The eluted fractions are fractionated by SDS-PAGE and
fractions containing the hsp90-peptide complexes identified
by immunoblotting using an anti-hsp90 antibody such as 3G3
(Affinity Bioreagents). Hsp90-peptide complexes can be
20 purified to apparent homogeneity using this procedure.
Typically, 150-200 µg of hsp90-peptide complex can be
purified from 1g of cells/tissue.

5.1.3. Preparation and Purification
of gp96-peptide Complexes

25 A procedure that can be used, presented by way of
example and not limitation, is as follows:

A pellet of tumors is resuspended in 3 volumes of buffer
consisting of 30mM sodium bicarbonate buffer (pH 7.5) and 1mM
30 PMSF and the cells allowed to swell on ice 20 minutes. The
cell pellet then is homogenized in a Dounce homogenizer (the
appropriate clearance of the homogenizer will vary according
to each cells type) on ice until >95% cells are lysed.

The lysate is centrifuged at 1,000g for 10 minutes to
35 remove unbroken cells, nuclei and other debris. The
supernatant from this centrifugation step then is
recentrifuged at 100,000g for 90 minutes. The gp96-peptide

complex can be purified either from the 100,000 pellet or from the supernatant.

When purified from the supernatant, the supernatant is diluted with equal volume of 2X lysis buffer and the

5 supernatant mixed for 2-3 hours at 4°C with Con a sepharose equilibrated with PBS containing 2mM Ca^{2+} and 2mM Mg^{2+}. Then, the slurry is packed into a column and washed with 1X lysis buffer until the OD_{280} drops to baseline. Then, the column is washed with 1/3 column bed volume of 10% α-methyl mannoside

10 (α-MM) dissolved in PBS containing 2mM Ca^{2+} and 2mM Mg^{2+}, the column sealed with a piece of parafilm, and incubated at 37°C for 15 minutes. Then the column is cooled to room temperature and the parafilm removed from the bottom of the column. Five column volumes of the α-MM buffer are applied

15 to the column and the eluate analyzed by SDS-PAGE. Typically the resulting material is about 60-95% pure; however, this depends upon the cell type and the tissue-to-lysis buffer ratio used. Then the sample is applied to a Mono Q FPLC column (Pharmacia) equilibrated with a buffer containing 5mM

20 sodium phosphate, pH 7. The proteins then are eluted from the column with a 0-1M NaCl gradient and the gp96 fraction elutes between 400mM and 550mM NaCl.

The procedure, however, may be modified by two additional steps, used either alone or in combination, to

25 consistently produce apparently homogeneous gp96-peptide complexes. One optional step involves an ammonium sulfate precipitation prior to the Con A purification step and the other optional step involves DEAE-Sepharose purification after the Con A purification step but before the Mono Q FPLC

30 step.

In the first optional step, the supernatant resulting from the 100,000g centrifugation step is brought to a final concentration of 50% ammonium sulfate by the addition of ammonium sulfate. The ammonium sulfate is added slowly while

35 gently stirring the solution in a beaker placed in a tray of ice water. The solution is stirred from about 1/2 to 12 hours at 4°C and the resulting solution centrifuged at 6,000

rpm (Sorvall SS34 rotor). The supernatant resulting from this step is removed, brought to 70% ammonium sulfate saturation by the addition of ammonium sulfate solution, and centrifuged at 6,000 rpm (Sorvall SS34 rotor). The resulting
5 pellet from this step is harvested and suspended in PBS containing 70% ammonium sulfate in order to rinse the pellet. This mixture is centrifuged at 6,000 rpm (Sorvall SS34 rotor) and the pellet dissolved in PBS containing 2mM Ca^{2+} and Mg^{2+}. Undissolved material is removed by a brief centrifugation at
10 15,000 rpm (Sorvall SS34 rotor). Then, the solution is mixed with Con A Sepharose and the procedure followed as before.

In the second optional step, the gp96 containing fractions eluted from the Con A column are pooled and the buffer exchanged for 5mM sodium phosphate buffer, pH 7, 300mM
15 NaCl by dialysis, or preferably by buffer exchange on a Sephadex G25 column. After buffer exchange, the solution is mixed with DEAE-Sepharose previously equilibrated with 5mM sodium phosphate buffer, pH 7, 300mM NaCl. The protein solution and the beads are mixed gently for 1 hour and poured
20 into a column. Then, the column is washed with 5mM sodium phosphate buffer, pH 7, 300mM NaCl, until the absorbance at 280mM drops to baseline. Then, the bound protein is eluted from the column with five volumes of 5mM sodium phosphate buffer, pH 7, 700mM NaCl. Protein containing fractions are
25 pooled and diluted with 5mM sodium phosphate buffer, pH 7 in order to lower the salt concentration to 175mM. The resulting material then is applied to the Mono Q FPLC column (Pharmacia) equilibrated with 5mM sodium phosphate buffer, pH 7 and the protein that binds to the Mono Q FPLC column
30 (Pharmacia) is eluted as described before.

It is appreciated, however, that one skilled in the art may assess, by routine experimentation, the benefit of incorporating the second optional step into the purification protocol. In addition, it is appreciated also that the
35 benefit of adding each of the optional steps will depend upon the source of the starting material.

When the gp96 fraction is isolated from the 100,000g
pellet, the pellet is suspended in 5 volumes of PBS
containing either 1% sodium deoxycholate or 1% oxtyl
glucopyranoside (but without the Mg^{2+} and Ca^{2+}) and incubated
5 on ice for 1 hour. The suspension is centrifuged at 20,000g
for 30 minutes and the resulting supernatant dialyzed against
several changes of PBS (also without the Mg^{2+} and Ca^{2+}) to
remove the detergent. The dialysate is centrifuged at
100,000g for 90 minutes, the supernatant harvested, and
10 calcium and magnesium are added to the supernatant to give
final concentrations of 2mM, respectively. Then the sample
is purified by either the unmodified or the modified method
for isolating gp96-peptide complex from the 100,000g
supernatant, see above.

15   The gp96-peptide complexes can be purified to apparent
homogeneity using this procedure. About 10-20µg of gp96 can
be isolated from 1g cells/tissue.

Infectious Disease

20   In an alternative embodiment wherein it is desired to
treat a patient having an infectious disease or prevent an
infectious disease, the above-described methods in Sections
5.1.1 - 5.1.3 are used to isolate hsp-peptide complexes from
cells infected with an infectious organism, e.g., of a cell
25 line or from a patient. Such infectious organisms include
but are not limited to, viruses, bacterial, protozoa, fungi,
and parasites as described in detail below.

30   ### 5.1.4. Isolation of Antigenic Molecules
         From Endogenous Complexes of
         Hsps or MHC Antigens

It has been found that antigenic peptides and/or
components can be eluted from endogenous, in vivo hsp-
complexes either in the presence of ATP or low pH. These
35 experimental conditions may be used to isolate peptides
and/or antigenic components from cells which may contain
potentially useful antigenic determinants. Once isolated,

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the amino acid sequence of each antigenic peptide may be
determined using conventional amino acid sequencing
methodologies. Such antigenic molecules can then be produced
by chemical synthesis or recombinant methods, purified, and
5 complexed to hsps *in vitro*.

Similarly, it has been found that potentially
immunogenic peptides may be eluted from MHC-peptide complexes
using techniques well known in the art (Falk, K. et al., 1990
Nature 348:248-251; Elliott, T., et al., 1990, *Nature*
10 348:195-197; Falk, K., et al., 1991, *Nature* 351:290-296).

Thus, potentially immunogenic or antigenic peptides may
be isolated from either endogenous stress protein-peptide
complexes or endogenous MHC-peptide complexes for use
subsequently as antigenic molecules, by complexing *in vitro*
15 to hsps. Exemplary protocols for isolating peptides and/or
antigenic components from either of the these complexes are
set forth below.

5.1.4.1   Peptides From Stress Protein-Peptide Complexes

20   Two methods may be used to elute the peptide from a
stress protein-peptide complex. One approach involves
incubating the stress protein-peptide complex in the presence
of ATP. The other approach involves incubating the complexes
in a low pH buffer.

25   Briefly the complex of interest is centrifuged through a
Centricon 10 assembly (Millipore) to remove any low molecular
weight material loosely associated with the complex. The
large molecular weight fraction may be removed and analyzed
by SDS-PAGE while the low molecular weight may be analyzed by
30 HPLC as described below. In the ATP incubation protocol, the
stress protein-peptide complex in the large molecular weight
fraction is incubated with 10mM ATP for 30 minutes at room
temperature. In the low pH protocol, acetic acid or
trifluoroacetic acid is added to the stress protein-peptide
35 complex to give a final concentration of 10% (vol/vol) and
the mixture incubated at room temperature in a boiling water
bath or any temperature in between for 10 minutes (*See*, Van

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Bleek, et al., 1990, *Nature* 348:213-216; and Li, et al., 1993, *EMBO Journal* 12:3143-3151).

The resulting samples are centrifuged through an Centricon 10 assembly as mentioned previously. The high and
5 low molecular weight fractions are recovered. The remaining large molecular weight stress protein-peptide complexes can be reincubated with ATP or low pH to remove any remaining peptides.

The resulting lower molecular weight fractions are
10 pooled, concentrated by evaporation and dissolved in 0.1% trifluoroacetic acid (TFA). The dissolved material is then fractionated by reverse phase high pressure liquid chromatography (HPLC) T using for example a VYDAC CIB reverse phase column equilibrated with 0.1% TFA. The bound material
15 is then eluted at a flow rate of about 0.8 ml/min by developing the column with a linear gradient of 0 to 80% acetonitrile in 0.1% TFA. The elution of the peptides can be monitored by OD_{210} and the fractions containing the peptides collected.
20

5.1.4.2 Peptides from MHC-peptide Complexes.

The isolation of potentially immunogenic peptides from MHC molecules is well known in the art and so is not described in detail herein (*See*, Falk, et al., 1990, *Nature*
25 348:248-251; Rotzsche, at al., 1990, *Nature* 348:252-254; Elliott, et al., 1990, *Nature* 348:191-197; Falk, et al., 1991, *Nature* 351:290-296; Demotz, et al., 1989, *Nature* 343:682-684; Rotzsche, et al., 1990, *Science* 249:283-287), the disclosures of which are incorporated herein by
30 reference.

Briefly, MHC-peptide complexes may be isolated by a conventional immunoaffinity procedure. The peptides then may be eluted from the MHC-peptide complex by incubating the complexes in the presence of about 0.1% TFA in acetonitrile.
35 The eluted peptides may be fractionated and purified by reverse phase HPLC, as before.

The amino acid sequences of the eluted peptides may be determined either by manual or automated amino acid sequencing techniques well known in the art. Once the amino acid sequence of a potentially protective peptide has been
5 determined the peptide may be synthesized in any desired amount using conventional peptide synthesis or other protocols well known in the art.

Peptides having the same amino acid sequence as those isolated above may be synthesized by solid-phase peptide
10 synthesis using procedures similar to those described by Merrifield, 1963, *J. Am. Chem. Soc.*, 85:2149. During synthesis, N-α-protected amino acids having protected side chains are added stepwise to a growing polypeptide chain linked by its C-terminal and to an insoluble polymeric
15 support i.e., polystyrene beads. The peptides are synthesized by linking an amino group of an N-α-deprotected amino acid to an α-carboxy group of an N-α-protected amino acid that has been activated by reacting it with a reagent such as dicyclohexylcarbodiimide. The attachment of a free
20 amino group to the activated carboxyl leads to peptide bond formation. The most commonly used N-α-protecting groups include Boc which is acid labile and Fmoc which is base labile.

Briefly, the C-terminal N-α-protected amino acid is
25 first attached to the polystyrene beads. The N-α-protecting group is then removed. The deprotected α-amino group is coupled to the activated α-carboxylate group of the next N-α-protected amino acid. The process is repeated until the desired peptide is synthesized. The resulting peptides are
30 then cleaved from the insoluble polymer support and the amino acid side chains deprotected. Longer peptides can be derived by condensation of protected peptide fragments. Details of appropriate chemistries, resins, protecting groups, protected amino acids and reagents are well known in the art and so are
35 not discussed in detail herein (*See*, Atherton, et al., 1989, *Solid Phase Peptide Synthesis: A Practical Approach*, IRL

Press, and Bodanszky, 1993, *Peptide Chemistry, A Practical Textbook*, 2nd Ed., Springer-Verlag).

Purification of the resulting peptides is accomplished using conventional procedures, such as preparative HPLC using
5 gel permeation, partition and/or ion exchange chromatography. The choice of appropriate matrices and buffers are well known in the art and so are not described in detail herein.

5.1.5 Exogenous Antigenic Molecules

10 Antigens or antigenic portions thereof can be selected for use as antigenic molecules, for complexing to hsps, from among those known in the art or determined by immunoassay to be able to bind to antibody or MHC molecules (antigenicity) or generate immune responses (immunogenicity). To determine
15 immunogenicity or antigenicity by detecting binding to antibody, various immunoassays known in the art can be used, including but not limited to, competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich"
20 immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in vivo* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, immunoprecipitation reactions, agglutination assays (e.*g*., gel agglutination
25 assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary
30 antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labelled. Many means are known in the art for detecting binding in an immunoassay and are envisioned for use. In one embodiment for detecting
35 immunogenicity, T cell-mediated responses can be assayed by standard methods, e.*g*., *in vitro* cytoxicity assays or *in vivo* delayed-type hypersensitivity assays.

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Potentially useful antigens or derivatives thereof for
use as antigenic molecules can also be identified by various
criteria, such as the antigen's involvement in neutralization
of a pathogen's infectivity (wherein it is desired to treat
5 or prevent infection by such a pathogen) (Norrby, 1985,
Summary, in Vaccines 85, Lerner, et al. (eds.), Cold Spring
Harbor Laboratory, Cold Spring Harbor, New York, pp.
388-389), type or group specificity, recognition by patients'
antisera or immune cells, and/or the demonstration of
10 protective effects of antisera or immune cells specific for
the antigen. In addition, where it is desired to treat or
prevent a disease caused by pathogen, the antigen's encoded
epitope should preferably display a small or no degree of
antigenic variation in time or amongst different isolates of
15 the same pathogen.

Preferably, where it is desired to treat or prevent
cancer, known tumor-specific antigens or fragments or
derivatives thereof are used. For example, such tumor
specific or tumor-associated antigens include, but are not
20 limited to, KS 1/4 pan-carcinoma antigen (Perez and Walker,
1990, *J. Immunol.* 142:3662-3667; Bumal, 1988, *Hybridoma*
7(4):407-415); ovarian carcinoma antigen (CA125) (Yu, et al.,
1991, *Cancer Res.* 51(2):468-475); prostatic acid phosphate
(Tailer, et al., 1990, *Nucl. Acids Res.* 18(16):4928);
25 prostate specific antigen (Henttu and Vihko, 1989, *Biochem.
Biophys. Res. Comm.* 160(2):903-910; Israeli, et al., 1993,
Cancer Res. 53:227-230); melanoma-associated antigen p97
(Estin, et al., 1989, *J. Natl. Cancer Inst.* 81(6):445-446);
melanoma antigen gp75 (Vijayasardahl, et al., 1990, *J. Exp.
30 Med.* 171(4):1375-1380); high molecular weight melanoma
antigen (Natali, et al., 1987, *Cancer* 59:55-63) and prostate
specific membrane antigen.

In a specific embodiment, an antigen or fragment or
derivative thereof specific to a certain tumor is selected
35 for complexing to hsp and subsequent sensitization of APC.

Preferably, where it is desired to treat or prevent
viral diseases, molecules comprising epitopes of known

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viruses are used as antigenic molecules. For example, such antigenic epitopes may be prepared from viruses including, but not limited to, hepatitis type A hepatitis type B, hepatitis type C, influenza, varicella, adenovirus, herpes simplex type I (HSV-I), herpes simplex type II (HSV-II), rinderpest, rhinovirus, echovirus, rotavirus, respiratory syncytial virus, papilloma virus, papova virus, cytomegalovirus, echinovirus, arbovirus, huntavirus, coxsachie virus, mumps virus, measles virus, rubella virus, polio virus, human immunodeficiency virus type I (HIV-I), and human immunodeficiency virus type II (HIV-II). Preferably, where it is desired to treat or prevent bacterial infections, molecules comprising epitopes of known bacteria are used. For example, such antigenic epitopes may be prepared from bacteria including, but not limited to, mycobacteria rickettsia, mycoplasma, neisseria and legionella.

Preferably, where it is desired to treat or prevent protozoal infectious, molecules comprising epitopes of known protozoa are used as antigenic molecules. For example, such antigenic epitopes may be prepared from protozoa including, but not limited to, leishmania, kokzidioa, and trypanosoma.

Preferably, where it is desired to treat or prevent parasitic infectious, molecules comprising epitopes of known parasites are used as antigenic molecules. For example, such antigenic epitopes may be from parasites including, but not limited to, chlamydia and rickettsia.

5.2   *In Vitro* Production of Stress Protein-Antigenic Molecule Complexes

In an embodiment in which complexes of hsps and the peptides with which they are endogenously associated *in vivo* are not employed, complexes of hsps to antigenic molecules are produced *in vitro*. As will be appreciated by those skilled in the art, the peptides either isolated by the aforementioned procedures or chemically synthesized or recombinantly produced may be reconstituted with a variety of naturally purified or recombinant stress proteins *in vitro* to

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generate immunogenic non-covalent stress protein-antigenic molecule complexes. Alternatively, exogenous antigens or antigenic/immunogenic fragments or derivatives thereof can be noncovalently complexed to stress proteins for use in the
5 immunotherapeutic or prophylactic vaccines of the invention. A preferred, exemplary protocol for noncovalently complexing a stress protein and an antigenic molecule *in vitro* is discussed below.

Prior to complexing, the hsps are pretreated with ATP or
10 low pH to remove any peptides that may be associated with the hsp of interest. When the ATP procedure is used, excess ATP is removed from the preparation by the addition of apyranase as described by Levy, et al., 1991, *Cell* 67:265-274. When the low pH procedure is used, the buffer is readjusted to
15 neutral pH by the addition of pH modifying reagents.

The antigenic molecules (1μg) and the pretreated hsp (9μg) are admixed to give an approximately 5 antigenic molecule: 1 stress protein molar ratio. Then, the mixture is incubated for 15 minutes to 3 hours at 4°-45° C in a suitable
20 binding buffer, for example, one containing 20mM sodium phosphate, pH 7.2, 350mM NaCl, 3mM $MgCl_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). The preparations are centrifuged through Centricon 10 assembly (Millipore) to remove any unbound peptide. The association of the peptides with the
25 stress proteins can be assayed by SDS-PAGE. This is the preferred method for *in vitro* complexing of peptides isolated from MHC-peptide complexes of peptides disassociated from endogenous hsp-peptide complexes.

In an alternative embodiment of the invention, preferred
30 for producing complexes of hsp70 to exogenous antigenic molecules such as proteins, 5-10 micrograms of purified hsp is incubated with equimolar quantities of the antigenic molecule in 20mM sodium phosphate buffer pH 7.5, 0.5M NaCl, 3mM $MgCl_2$ and 1mM ADP in a volume of 100 microliter at 37°C
35 for 1 hr. This incubation mixture is further diluted to 1ml in phosphate-buffered saline.

In an alternative embodiment of the invention, preferred for producing complexes of gp96 or hsp90 to peptides, 5-10 micrograms of purified gp96 or hsp90 is incubated with equimolar or excess quantities of the antigenic peptide in a
5 suitable buffer such as one containing 20mM sodium phosphate buffer, pH 7.5, 0.5M NaCl, 3mM MgCl$_2$ at 60°-65°C for 5-20 minutes. This incubation mixture is allowed to cool to room temperature and centrifuged more than once, if necessary through Centricon 10 assembly (Millipore) to remove any
10 unbound peptide.

Following complexing, the stress protein-antigenic molecule complexes can optionally be assayed *in vitro* for immunogenicity, using for example the mixed lymphocyte target cell assay (MLTC) described below.
15

5.2.1 Determination of Immunogenicity of Stress Protein-Peptide Complexes

The purified stress protein-antigenic molecule complexes can optionally be assayed for immunogenicity using the mixed
20 lymphocyte target culture assay (MLTC) well known in the art, prior to their use to sensitize APC. It will be understood that this procedure is entirely optional, and not necessary to practice the present invention.

By way of example but not limitation, the following
25 procedure can be used. Briefly, mice are injected subcutaneously with the candidate stress protein-antigenic molecule complexes. Other mice are injected with either other stress protein peptide complexes or whole infected cells which act as positive controls for the assay. The mice
30 are injected twice, 7-10 days apart. Ten days after the last immunization, the spleens are removed and the lymphocytes released. The released lymphocytes may be restimulated subsequently *in vitro* by the addition of dead cells that expressed the complex of interest.

35   For example, 8x10^6 immune spleen cells may be stimulated with 4x10^4 mitomycin C treated or γ-irradiated (5-10,000 rads) infected cells (or cells transfected with an appropriate

gene, as the case may be) in 3ml RPMI medium containing 10%
fetal calf serum. In certain cases 33% secondary mixed
lymphocyte culture supernatant may be included in the culture
medium as a source of T cell growth factors (See, Glasebrook,
5 et al., 1980, J. Exp. Med. 151:876). To test the primary
cytotoxic T cell response after immunization, spleen cells
may be cultured without stimulation. In some experiments
spleen cells of the immunized mice may also be restimulated
with antigenically distinct cells, to determine the
10 specificity of the cytotoxic T cell response.

Six days later the cultures are tested for cytotoxicity
in a 4 hour ^{51}Cr-release assay (See, Palladino, et al., 1987,
Cancer Res. 47:5074-5079 and Blachere, at al., 1993, J.
Immunotherapy 14:352-356). In this assay, the mixed
15 lymphocyte culture is added to a target cell suspension to
give different effector:target (E:T) ratios (usually 1:1 to
40:1). The target cells are prelabelled by incubating 1x10^6
target cells in culture medium containing 200 mCi ^{51}Cr/ml for
one hour at 37°C. The cells are washed three times following
20 labeling. Each assay point (E:T ratio) is performed in
triplicate and the appropriate controls incorporated to
measure spontaneous ^{51}Cr release (no lymphocytes added to
assay) and 100% release (cells lysed with detergent). After
incubating the cell mixtures for 4 hours, the cells are
25 paletted by centrifugation at 200g for 5 minutes. The amount
of ^{51}Cr released into the supernatant is measured by a gamma
counter. The percent cytotoxicity is measured as cpm in the
test sample minus spontaneously released cpm divided by the
total detergent released cpm minus spontaneously released
30 cpm.

In order to block the MHC class I cascade a concentrated
hybridoma supernatant derived from K-44 hybridoma cells (an
anti-MHC class I hybridoma) is added to the test samples to a
final concentration of 12.5%.
35

5.3. Obtaining Macrophages and
Antigen-Presenting Cells

The antigen-presenting cells, including but not limited
to macrophages, dendritic cells and B-cells, are preferably
5 obtained by production *in vitro* from stem and progenitor
cells from human peripheral blood or bone marrow as described
by Inaba, K., et al., 1992, J. Exp. Med. 176:1693-1702.

APC can be obtained by any of various methods known in
the art. In a preferred aspect human macrophages are used,
10 obtained from human blood cells. By way of example but not
limitation, macrophages can be obtained as follows:

Mononuclear cells are isolated from peripheral blood of
a patient (preferably the patient to be treated), by Ficoll-
Hypaque gradient centrifugation.

15 Tissue culture dishes are pre-coated with the patient's
own serum or with other AB+ human serum and incubated at 37°C
for 1 hr. Non-adherent cells are removed by pipetting. To
the adherent cells left in the dish, is added cold (4°C) 1mM
EDTA in phosphate-buffered saline and the dishes are left at
20 room temperature for 15 minutes. The cells are harvested,
washed with RPMI buffer and suspended in RPMI buffer.
Increased numbers of macrophages may be obtained by
incubating at 37°C with macrophage-colony stimulating factor
(M-CSF); increased numbers of dendritic cells may be obtained
25 by incubating with granulocyte-macrophage-colony stimulating
factor (GM-CSF) as described in detail by Inaba, K., et al.,
1992, J. Exp. Med. 176:1693-1702.

5.4. Sensitization of Macrophages
and Antigen Presenting Cells
30 ### With Hsp-Complexes

APC are sensitized with hsp non-covalently bound to
antigenic molecules by incubating the cells *in vitro* with the
complexes. The APC are sensitized with complexes of hsps and
antigenic molecules by incubating *in vitro* with the hsp-
35 complex at 37°C for 15 min.-24 hrs. By way of example but
not limitation, 4×10^7 macrophages can be incubated with 10

microgram gp96-peptide complexes per ml or 100 microgram
hsp90-peptide complexes per ml at 37°C for 15 min.-24 hrs. in
1 ml plain RPMI medium. The cells are washed three times and
resuspended in a physiological medium preferably sterile, at
5 a convenient concentration (e.g., $1x10^7$/ml) for infusion in a
patient. Preferably, the patient into which the sensitized
APCs are infused is the patient from which the APC were
originally isolated (autologous embodiment).

Optionally, the ability of sensitized APC to stimulate,
10 for example, the antigen-specific, class I - restricted
cytotoxic T-lymphocytes (CTL) can be monitored by their
ability to stimulate CTLs to release tumor neurosis factor,
and by their ability to act as targets of such CTLs, as
described below in Example 6, below.
15

5.5. Reinfusion of Sensitized APC
The hsp-sensitized macrophages and other APC are
reinfused into the patient systemically, preferably
intravenously, by conventional clinical procedures. These
20 activated cells are reinfused, preferentially by systemic
administration into the autologous patient. Patients
generally receive from about 10^6 to about 10^{12} sensitized
macrophages, depending on the condition of the patient. In
some regimens, patients may optionally receive in addition a
25 suitable dosage of a biological response modifier including
but not limited to the cytokines IFN-α, IFN-α, IL-2, IL-4,
IL-6, TNF or other cytokine growth factor.

5.6 Target Infectious Diseases
30 Infectious diseases that can be treated or prevented by
the methods of the present invention are caused by infectious
agents including but not limited to viruses, bacteria, fungi
protozoa and parasites.

Viral diseases that can be treated or prevented by the
35 methods of the present invention include, but are not limited
to, those caused by hepatitis type A, hepatitis type B,
hepatitis type C, influenza, varicella, adenovirus, herpes

simplex type I (HSV-I), herpes simplex type II (HSV-II), rinderpest, rhinovirus, echovirus, rotavirus, respiratory syncytial virus, papilloma virus, papova virus, cytomegalovirus, echinovirus, arbovirus, huntavirus,
5 coxsachie virus, mumps virus, measles virus, rubella virus, polio virus, human immunodeficiency virus type I (HIV-I), and human immunodeficiency virus type II (HIV-II).

Bacterial diseases that can be treated or prevented by the methods of the present invention are caused by bacteria
10 including, but not limited to, mycobacteria rickettsia, mycoplasma, neisseria and legionella.

Protozoal diseases that can be treated or prevented by the methods of the present invention are caused by protozoa including, but not limited to, leishmania, kokzidioa, and
15 trypanosoma.

Parasitic diseases that can be treated or prevented by the methods of the present invention are caused by parasites including, but not limited to, chlamydia and rickettsia.

20   5.7. **Target Cancers**

Cancers that can be treated or prevented by the methods of the present invention include, but not limited to human sarcomas and carcinomas, e.g., fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma,
25 angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma,
30 adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma,
35 Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma,

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craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma; leukemias, e.g., acute lymphocytic leukemia and acute myelocytic leukemia

5 (myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia); chronic leukemia (chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia); and polycythemia vera, lymphoma (Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenström's

10 macroglobulinemia, and heavy chain disease. Specific examples of such cancers are described in the sections below.

In a specific embodiment the cancer is metastatic. In another embodiment, the cancer is a tumor. In another specific embodiment, the patient having a cancer is

15 immunosuppressed by reason of having undergone anti-cancer therapy (e.g., chemotherapy radiation) prior to administration of the hsp-sensitized APC.

5.7.1.   Colorectal Cancer
20   Metastatic to the Liver

In 1992, approximately 150,000 Americans were diagnosed with colorectal cancer and more than 60,000 died as a result of colorectal metastases. At the time of their deaths, 80 percent of patients with colorectal cancer have metastatic

25 disease involving the liver, and one-half of these patients have no evidence of other (extrahepatic) metastases. Most metastatic tumors of the liver are from gastrointestinal primaries. Unfortunately, the natural history of metastatic liver lesions carries a grave prognosis and systemic

30 chemotherapy regimens have been unable to induce significant response rates or alter length of survival (Drebin, J.A., et al., in *Current Therapy In Oncology*, ed. J.E. Niederhuber, B.C. Decker, Mosby, 1993, p.426).

Colorectal cancer initially spreads to regional lymph
35 nodes and then through the portal venous circulation to the liver, which represents the most common visceral site of metastasis. The symptoms that lead patients with colorectal

cancer to seek medical care vary with the anatomical location of the lesion. For example, lesions in the ascending colon frequency ulcerate, which leads to chronic blood loss in the stool.

5   Radical resection offers the greatest potential for cure in patients with invasive colorectal cancer. Before surgery, the CEA titer is determined. Radiation therapy and chemotherapy are used in patients with advanced colorectal cancer. Results with chemotherapeutic agents (e.g., 5-

10 fluorouracil) are mixed and fewer than 25 percent of patients experience a greater than 50 percent reduction in tumor mass (Richards, 2d., F., et al., 1986, J. Clin. Oncol. 4:565).

Patients with widespread metastases have limited survival and systemic chemotherapy has little impact in this

15 group of patients. In addition, systemically administered chemotherapy is often limited by the severity of toxicities associated with the various agents, such as severe diarrhea, mucositis and/or myelosuppression. Other techniques, including hepatic radiation, systemic chemotherapy, hepatic

20 arterial ligation, tumor embolization and immunotherapy have all been explored, but, for the most part, have proven ineffectual in prolonging patient survival.

In a specific embodiment, the present invention provides compositions and methods for enhancing tumor specific

25 immunity in individuals suffering from colorectal cancer metastasized to the liver, in order to inhibit the progression of the neoplastic disease. Preferred methods of treating these neoplastic diseases comprise administering a composition of autologous macrophage that have been

30 stimulated with hsp noncovalently bound to peptide complexes, which elicits tumor-specific immunity against the tumor cells. In a specific embodiment, the hsp-stimulated APC of the invention are used to inhibit liver cancer growth in cancer patients, without inducing toxicity.

35   Accordingly, as an example of the method of the invention, hsp-stimulated APC are administered to a patient

diagnosed with colorectal cancer, with or without liver
metastasis, by intravenous injection.

5.7.2. Hepatocellular Carcinoma

5   Hepatocellular carcinoma is generally a disease of the
elderly in the United States. Although many factors may lead
to hepatocellular carcinoma, the disease is usually limited
to those persons with preexisting liver disease.
Approximately 60 to 80 percent of patients in the United

10 States with hepatocellular carcinoma have a cirrhotic liver
and about four percent of individuals with a cirrhotic liver
eventually develop hepatocellular carcinoma (Niederhuber,
J.E., (ed.), 1993, Current Therapy in Oncology, B.C. Decker,
Mosby). The risk is highest in patients whose liver disease

15 is caused by inherited hemochromatosis or hepatic B viral
infection (Bradbear, R.A., et al., 1985, J. Natl. Cancer
Inst. 75:81; Beasley, R.P., et al., 1981, Lancet 2:1129).
Other causes of cirrhosis that can lead to hepatocellular
carcinoma include alcohol abuse and hepatic fibrosis caused

20 by chronic administration of methotrexate. The most frequent
symptoms of hepatocellular carcinoma are the development of a
painful mass in the right upper quadrant or epigastrium,
accompanied by weight loss. In patients with cirrhosis, the
development of hepatocellular carcinoma is preceded by

25 ascites, portal hypertension and relatively abrupt clinical
deterioration. In most cases, abnormal values in standard
liver function tests such as serum aminotransferase and
alkaline phosphatase are observed.

CT scans of the liver are used to determine the anatomic
30 distribution of hepatocellular carcinoma and also provide
orientation for percutaneous needle biopsy. Approximately 70
percent of patients with hepatocellular carcinoma have an
elevated serum alpha-fetoprotein concentration (McIntire,
K.R., et al., 1975, Cancer Res. 35:991) and its concentration
35 correlates with the extent of the disease.

Radical resection offers the only hope for cure in
patients with hepatocellular carcinoma. Such operative

- 33 -

procedures are associated with five-year survival rates of 12
to 30 percent. Liver transplantation may improve survival of
some younger individuals. However, most patients are not
surgical candidates because of extensive cirrhosis multifocal
5 tumor pattern or scarcity of compatible donor organs.
Chemotherapeutic agents have been administered either by
intravenous route or through an intrahepatic arterial
catheter. Such therapy has sometimes been combined with
irradiation to the liver. Reductions in the size of
10 measurable tumors of 50% or more have been reported in some
patients treated with either systemic doxorubicin or 5-
fluorouracil. However, chemotherapy often induces
immunosuppression and rarely causes the tumor to disappear
completely and the duration of response is short. The
15 prognosis for patients with hepatocellular carcinoma is
negatively correlated with cirrhosis and metastases to the
lungs or bone. Median survival for patients is only four to
six months. In another specific embodiment, the present
invention provides compositions and methods for enhancing
20 specific immunity in individuals suffering from
hepatocellular carcinoma in order to inhibit the progression
of the neoplastic disease and ultimately eliminate all
preneoplastic an neoplastic cells.

25   **5.7.3. Breast Cancer**
Another specific aspect of the invention relates to the
treatment of breast cancer. The American Cancer Society
estimated that in 1992 180,000 American women were diagnosed
with breast cancer and 46,000 succumbed to the disease
30 (Niederhuber, J.E.ed. *Current Therapy in Oncology* B.C.
Decker, Mosby, 1993). This makes breast cancer the second
major cause of cancer death in women, ranking just behind
lung cancer. A disturbing fact is the observation that
breast cancer has been increasing at a rate of 3 percent per
35 year since 1980 (Niederhuber, J.E., ed. Current Therapy in
Oncology, B.C. Decker, Mosby, (1993)). The treatment of
breast cancer presently involves surgery, radiation, hormonal

therapy and/or chemotherapy. Consideration of two breast
cancer characteristics, hormone receptors and disease extent,
has governed how hormonal therapies and standard-dose
chemotherapy are sequenced to improve survival and maintain
5 or improve quality of life. A wide range of multidrug
regimens have been used as adjuvant therapy in breast cancer
patients, including, but not limited to combinations of 2
cyclophosphamide, doxorubicin, vincristine methotrexate, 5-
fluorouracil and/or leucovorin. In a specific embodiment,
10 the present invention provides hsp compositions and methods
for enhancing specific immunity to preneoplastic and
neoplastic mammary cells in women. The present invention
also provides compositions and methods for preventing the
development of neoplastic cells in women at enhanced risk for
15 breast cancer, and for inhibiting cancer cell proliferation
and metastasis. These compositions can be applied alone or
in combination with each other or with biological response
modifiers.

20   5.8. **Autologous Embodiment**
        In a preferred embodiment of the invention directed to
the use of autologous APC stimulated with autologous
complexes of hsp-peptides for the treatment or prevention of
cancer, two of the most intractable hurdles to cancer
25 immunotherapy are circumvented. First is the possibility
that human cancers, like cancers of experimental animals, are
antigenically distinct. In a preferred embodiment of the
present invention, hsps chaperone antigenic peptides of the
cancer cells from which they are derived and circumvent this
30 hurdle. Second, most current approaches to cancer
immunotherapy focus on determining the CTL-recognized
epitopes of cancer cell lines. This approach requires the
availability of cell lines and CTLs against cancers. These
reagents are unavailable for an overwhelming proportion of
35 human cancers. In an embodiment of the present invention
directed to the use of autologous macrophages stimulated with
autologous complexes comprising hsp peptides, cancer

- 35 -

immunotherapy does not depend on the availability of cell lines or CTLs nor does it require definition of the antigenic epitopes of cancer cells. These advantages make autologous hsp-stimulated APC attractive and novel immunogens against
5 cancer.

5.9. Prevention and Treatment of Primary and Metastatic Neoplastic Diseases

There are many reasons why immunotherapy as provided by
10 the present invention is desired for use in cancer patients. First, if cancer patients are immunosuppressed and surgery, with anesthesia, and subsequent chemotherapy, may worsen the immunosuppression, then with appropriate immunotherapy in the preoperative period, this immunosuppression may be prevented
15 or reversed. This could lead to fewer infectious complications and to accelerated wound healing. Second, tumor bulk is minimal following surgery and immunotherapy is most likely to be effective in this situation. A third reason is the possibility that tumor cells are shed into the
20 circulation at surgery and effective immunotherapy applied at this time can eliminate these cells.

In a specific embodiment, the preventive and therapeutic methods of the invention are directed at enhancing the immunocompetence of the cancer patient either before surgery,
25 at or after surgery, and to induce tumor-specific immunity to cancer cells, with the objective being inhibition of cancer, and with the ultimate clinical objective being total cancer regression and eradication.

30   ## 5.10. Monitoring of Effects After Adoptive Immunotherapy

The effect of immunotherapy with hsp-stimulated APC on development and progression of neoplastic diseases can be monitored by any methods known to one skilled in the art, including but not limited to measuring: a) delayed
35 hypersensitivity as an assessment of cellular immunity; b) activity of cytolytic T-lymphocytes *in vitro*; c) levels of

tumor specific antigens, e.g., carcinoembryonic (CEA)
antigens; d) changes in the morphology of tumors using
techniques such as a computed tomographic (CT) scan; e)
changes in levels of putative biomarkers of risk for a
5 particular cancer in individuals at high risk; and f) changes
in the morphology of tumors using a sonogram.

5.10.1.   Delayed Hypersensitivity Skin Test

Delayed hypersensitivity skin tests are of great value
10 in the overall immunocompetence and cellular immunity to an
antigen. Inability to react to a battery of common skin
antigens is termed anergy (Sato, T., et al, 1995, *Clin.
Immunol. Pathol.* 74:35-43).

Proper technique of skin testing requires that the
15 antigens be stored sterile at 4°C, protected from light and
reconstituted shorted before use. A 25- or 27-gauge need
ensures intradermal, rather than subcutaneous, administration
of antigen. Twenty-four and 48 hours after intradermal
administration of the antigen, the largest dimensions of both
20 erythema and induration are measured with a ruler.
Hypoactivity to any given antigen or group of antigens is
confirmed by testing with higher concentrations of antigen
or, in ambiguous circumstances, by a repeat test with an
intermediate test.
25

5.10.2.   Activity of Cytolytic
T-lymphocytes *In Vitro*

Eight x 10^6 peripheral blood derived T lymphocytes
isolated by the Ficoll-Hypaque centrifugation gradient
30 technique, are restimulated with 4x10^4 mitomycin C treated
tumor cells in 3ml RPMI medium containing 10% fetal calf
serum. In some experiments, 33% secondary mixed lymphocyte
culture supernatant or IL-2, is included in the culture
medium as a source of T cell growth factors.

35   In order to measure the primary response of cytolytic T-
lymphocytes after immunization, T cells are cultured without
the stimulator tumor cells. In other experiments, T cells

are restimulated with antigenically distinct cells. After
six days, the cultures are tested for cytotoxity in a 4 hour
^{51}Cr-release assay. The spontaneous ^{51}Cr-release of the
targets should reach a level less than 20%. For the anti-MHC
5 class I blocking activity, a tenfold concentrated supernatant
of W6/32 hybridoma is added to the test at a final
concentration of 12.5% (Heike M., et al., J. *Immunotherapy*
15:165-174).

10   **5.10.3. Levels of Tumor Specific Antigens**
          Although it may not be possible to detect unique tumor
antigens on all tumors, many tumors display antigens that
distinguish them from normal cells. The monoclonal antibody
reagents have permitted the isolation and biochemical
15 characterization of the antigens and have been invaluable
diagnostically for distinction of transformed from
nontransformed cells and for definition of the cell lineage
of transformed cells. The best-characterized human tumor-
associated antigens are the oncofetal antigens. These
20 antigens are expressed during embryogenesis, but are absent
or very difficult to detect in normal adult tissue. The
prototype antigen is carcinoembryonic antigen (CEA), a
glycoprotein found on fetal gut an human colon cancer cells,
but not on normal adult colon cells. Since CEA is shed from
25 colon carcinoma cells and found in the serum, it was
originally thought that the presence of this antigen in the
serum could be used to screen patients for colon cancer.
However, patients with other tumors, such as pancreatic and
breast cancer, also have elevated serum levels of CEA.
30 Therefore, monitoring the fall and rise of CEA levels in
cancer patients undergoing therapy has proven useful for
predicting tumor progression and responses to treatment.
          Several other oncofetal antigens have been useful for
diagnosing and monitoring human tumors, e.g., alpha-
35 fetoprotein, an alpha-globulin normally secreted by fetal
liver and yolk sac cells, is found in the serum of patients

with liver and germinal cell tumors and can be used as a
matter of disease status.

5.10.4. Computed Tomographic (CT) Scan

5   CT remains the choice of techniques for the accurate
staging of cancers. CT has proved more sensitive and
specific than any other imaging techniques for the detection
of metastases.

10   ### 5.10.5. Measurement of Putative Biomarkers
The levels of a putative biomarker for risk of a
specific cancer are measured to monitor the effect of hsp
noncovalently bound to peptide complexes. For example, in
individuals at enhanced risk for prostate cancer, serum
15 prostate-specific antigen (PSA) is measured by the procedure
described by Brawer, M.K., et. al., 1992, *J. Urol.* 147:841-
845, and Catalona, W.J., et al., 1993, *JAMA* 270:948-958; or
in individuals at risk for colorectal cancer CEA is measured
as described above in Section 4.5.3; and in individuals at
20 enhanced risk for breast cancer, 16-α-hydroxylation of
estradiol is measured by the procedure described by
Schneider, J. et al., 1982, *Proc. Natl. Acad. Sci. USA*
79:3047-3051. The references cited above are incorporated by
reference herein in their entirety.
25

5.10.6. Sonogram
A Sonogram remains an alternative choice of technique
for the accurate staging of cancers.

30   ### 6. EXAMPLES: EXOGENOUS HEAT SHOCK PROTEIN-ASSOCIATED PEPTIDES ARE RE-PRESENTED BY MHC I MOLECULES OF MACROPHAGE
The possibility of re-presentation of HSP-chaperoned
peptides by phagocytic cells was tested directly using a
vesicular stomatitis virus (VSV) model, for example, the VSV-
35 infected cell line, EL4 cells.

6.1. Methods and Materials

Gp96 was isolated to apparent homogeneity from EL4 cells transfected with the gene encoding the nucleocapsid protein of VS1 (N1 cells) and from untransfected EL4 cells (negative
5 control), according to procedures described above in Section 5.

Macrophages were prepared from 0.2ml pristane-induced peritoneal exudate cells from C57BL/6 mice.

10
6.2. Ability of Sensitised Macrophages to Stimulate TNF Release by CTLs

1×10^4 macrophages were cultured in the presence of gp96 (2 or 10 microgram/ml derived from N1 cells or EL4 cells) and VSV peptide-specific CTL (5×10^4) in 96-well U-bottom plate
15 at 37°C. After 24 hr supernatants were collected and TNF-α product was measured by bioassay in a cytotoxicity assay using WEHI164 cells.

WEHI164 cells (2.5×10^2/well) were cocultured with serially diluted supernatants obtained previously as
20 described above. α-TNF-α was cultured with WEHI164 cells in separate wells as control. After a 4 hr incubation at 37°C, 50μl 3-[4,5-dimethyl thiazol-2-yl]- 2,5 diphyl tetrazolium bromide (MTT) (SIGMA, St. Louis) (1mg/ml) was added and incubated for a further 4 hr. 100 microliter of propanol -
25 0.05 MHCl was added and the optic density was measured immediately at 590 nm. Sample concentrations were calculated by comparison with dilution points which resulted in killing of 50% of WEHI164 cells.

30
6.2.1. Results

Macrophages sensitized with gp96 isolated from N1 cells stimulated release of TNF by VSV-specific CTLs, while those sensitized with gp96 isolated from EL4 cells did not (Figure 1A).

35

6.3. Ability of Sensitised Macrophages
to Act As Targets of CTLs

Peritoneal macrophages (5×10^6) were sensitized with gp96 (10 micrograms/ml) derived from N1 cells, EL4 cells, VSV
5 nucleocapsid K^b epitope peptide (10 micro M) as a positive control, or culture medium as control, for 2 hr at 37°C, followed by labeling with ^{51}Cr for 1.5 hr. These sensitized macrophages were used as targets in a 4 hr ^{51}Cr-release assay with VSV-specific CTLs.
10   Anti-CD4 mAb (CK1.5), anti-CD8 mAb (YTS169.4), anti-H-2K^b mAb (Y3), anti-H-2D^b mAb (B22.249) or RPMI control were added to the CTL assay at the same time as effector cells and ^{51}Cr-labeled macrophages sensitized with gp96 from N1 cells.

15   6.3.1. Results

Macrophages sensitized with gp96 isolated from N1 cells were lysed by anti-VSV CTLs whereas those sensitized with gp96 from EL4 cells were not (Figure 1B). The lysis is blocked by anti-MHC class I (Jb) and anti-CD8 antibodies but
20 not by anti-MHC class I (D6) and anti-CD4 antibodies (Figure 1C).

Thus, gp96 molecules isolated from N1 cells chaperone the Kb-epitope of VSV and this epitope is inducted into the endogenous presentation pathway of macrophages.
25

7.   EXAMPLES: ADOPTIVE TRANSFER OF SENSITIZED
MACROPHAGES

7.1.   Materials and Methods

30   Peritoneal macrophages were collected three days after injecting nominally healthy BALB/cJ mice with 0.2ml pristane (Sigma, St. Louis) intraperitoneally, by centrifuging the peritoneal exudate.

Peritoneal exudate cells (PEC) or macrophages (4×10^7)
35 were incubated at 37°C for 3 hr in 1ml RPMI containing 50µg gp96-peptide complexes derived from Methylcholanthrene-induced tumors or from liver. The macrophages were then

washed 3 times and resuspended at a concentrate of 1×10^7/ml in
RPMI medium.   200 microliter of this suspension was used to
inject mice intraperitoneally as described in the
experimental protocol below.
5

7.2. Methylcholanthrene (Meth A)-
Induced Sarcoma Model

Five groups of mice were given the following treatments:
a) Subcutaneous infection of buffer solution; b) Subcutaneous
10 infection of 9μg gp96-peptide complexes derived from liver
tissue; c) Intraperitoneal infection with 5×10^6 PEC or
macrophages sensitized with gp96-peptide complexes derived
from normal liver; d) Subcutaneous injection containing 9μg
gp96-peptide complexes derived from Meth A tumor cells; and
15 e) Intraperitoneal injection with 5×10^6 PEC or macrophages
sensitized with gp96 peptide complexes derived from Meth A
tumor cells.

The above treatment regimes were administered twice, at
weekly intervals before injecting intradermally, 1 week after
20 the second injection, 1×10^5 Meth A tumor cells. Tumor growth
was monitored by measuring the average tumor diameter.

7.3. Results

Tumor growth was comparable in groups A, B and C, i.e.
25 mice receiving the control buffer solution or the gp96
derived from liver tissue. In mice treated directly with
gp96-peptide complexes (D) or with gp96-peptide complex
sensitized macrophages, (E) tumor growth was markedly
inhibited compared with the mice receiving the buffer control
30 or the gp96 derived from liver (Figure 2). Thus,
administration of gp96-peptide complexes directly or adoptive
therapy with macrophages and/or other APC sensitized with
gp96-peptide complexes, described herein, represents an
approach to treatment of cancer with potential applicability
35 to a wide range of cancers, infectious diseases or
immunological disorders.

The present invention is not to be limited in scope by
the specific embodiments described herein. Indeed, various
modifications of the invention in addition to those described
herein will become apparent to those skilled in the art from

5 the foregoing description and accompanying figures. Such
modifications are intended to fall within the scope of the
appended claims.

Various publications are cited herein, the disclosures
of which are incorporated by reference in their entireties.

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WHAT IS CLAIMED IS:

1. A method of treating an individual having cancer comprising the steps of:
    (a) sensitizing antigen presenting cells *in vitro* with a sensitizing- effective amount of a complex of a heat shock protein noncovalently bound to an antigenic molecule; and
    (b) administering to the individual a therapeutically effective amount of the sensitized antigen presenting cells.

2. A method of treating an individual having cancer comprising administering to the individual a therapeutically effective amount of sensitized antigen presenting cells, in which the antigen presenting cells have been sensitized *in vitro* with a complex of a heat shock protein noncovalently bound to an antigenic molecule.

3. The method according to claim 1 wherein the individual is human.

4. The method according to claim 2 wherein the individual is human.

5. The method according to claim 3 wherein the antigen presenting cells are autologous to the human.

6. The method according to claim 4 wherein the antigen presenting cells are autologous to the human.

7. The method according to claim 3 wherein the antigen presenting cells are allogeneic to the human.

8. The method according to claim 4 wherein the antigen presenting cells are allogeneic to the human.

9.   The method according to claim 3 or 4 wherein the
cancer is selected from the group consisting of fibrosarcoma,
myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma,
chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma,
5 lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's
tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma,
pancreatic cancer, breast cancer, ovarian cancer, prostate
cancer, squamous cell carcinoma, basal cell carcinoma,
adenocarcinoma, sweat gland carcinoma, sebaceous gland
10 carcinoma, papillary carcinoma, papillary adenocarcinomas,
cystadenocarcinoma, medullary carcinoma, bronchogenic
carcinoma, renal cell carcinoma, hepatoma, bile duct
carcinoma, choriocarcinoma, seminoma, embryonal carcinoma,
Wilms' tumor, cervical cancer, testicular tumor, lung
15 carcinoma, small cell lung carcinoma, bladder carcinoma,
epithelial carcinoma, glioma, astrocytoma, medulloblastoma,
craniopharyngioma, ependymoma, pinealoma, hemangioblastoma,
acoustic neuroma, oligodendroglioma, meningioma, melanoma,
neuroblastoma, retinoblastoma, leukemia, lymphoma, multiple
20 myeloma, Waldenström's macroglobulinemia, and heavy chain
disease.

10.   The method according to claim 3 or 4 wherein the
heat shock protein is selected from the group consisting of
25 hsp70, hsp90, gp96, and a combination thereof.

11.   The method according to claim 4 wherein the heat
shock protein is hsp70.

30   12.   The method of according to claim 4 wherein the heat
shock protein is hsp90.

13.   The method according to claim 4 wherein the heat
shock protein is gp96.
35

14.   The method according to claim 4 wherein the complex
is isolated from a tumor autologous to the human.

15.   The method according to claim 4 wherein the complex is isolated from a tumor allogeneic to the human.

16.   The method according to claim 4 further comprising
5 administering to the human an effective amount of a biological response modifier selected from the group consisting of interferon-α, interferon-α, interleukin-2, interleukin-4, interleukin-6 and tumor necrosis factor.

10   17.   The method according to claim 4 wherein the therapeutically effective amount is in the range of 10^6 to 10^{12} cells.

18.   The method according to claim 4 wherein the cells
15 are administered intravenously.

19.   The method according to claim 4 wherein the complex is isolated from cells of said cancer of the individual.

20   20.   The method according to claim 4 wherein the antigenic molecule is an exogenous antigenic molecule.

21.   The method according to claim 20 in which the exogenous antigenic molecule is a tumor antigen.
25

22.   A method of treating or preventing an infectious disease in an individual in whom such treatment or prevention is desired comprising the steps of:

    (a)   sensitizing antigen presenting cells *in vitro*
30   with a sensitizing-effective amount of a complex of a heat shock protein noncovalently bound to an antigenic molecule; and

    (b)   administering to the individual a therapeutically effective amount of the
35   sensitized antigen presenting cells.

23. A method of treating or preventing an infectious disease in an individual in whom such treatment or prevention is desired comprising administering to the individual a therapeutically effective amount of sensitized antigen
5 presenting cells, in which the antigen presenting cells have been sensitized *in vitro* with a complex of a heat shock protein noncovalently bound to an antigenic molecule.

24. The method according to claim 22 wherein the
10 individual is human.

25. The method according to claim 23 wherein the individual is human.

15   26. The method according to claim 24 wherein the antigen presenting cells are autologous to the human.

27. The method according to claim 25 wherein the antigen presenting cells are autologous to the human.
20
28. The method according to claim 24 wherein the antigen presenting cells are allogeneic to the human.

29. The method according to claim 25 wherein the
25 antigen presenting cells are allogeneic to the human.

30. The method according to claim 25 wherein the complex is isolated from a cell infected with an infectious agent that causes the infectious disease.
30
31. The method according to claim 30 wherein the cell is autologous to the human.

32. The method according to claim 25 wherein the
35 antigenic molecule is an exogenous antigenic molecule.

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33. The method according to claim 32 wherein the exogenous antigenic molecule is an antigen of an infectious agent that causes the infectious disease.

5   34. The method according to claim 25 wherein the infectious disease is caused by a virus, bacterium, fungus, parasite, or protozoa.

35. The method according to claim 25 wherein the heat
10 shock protein is selected from the group consisting of hsp70, hsp90, gp96 and a combination thereof.

36. A pharmaceutical composition comprising a therapeutically effective amount of sensitized antigen
15 presenting cells, in a pharmaceutically acceptable carrier, in which the antigen presenting cells have been sensitized *in vitro* with a complex of a heat shock protein noncovalently bound to an antigenic molecule.

20   37. The composition of claim 36 in which the antigen presenting cells are human.

38. The composition of claim 37 wherein the heat shock protein is selected from the group consisting of hsp70,
25 hsp90, gp96 and a combination thereof.

39. A kit comprising in a container a complex of a heat shock protein noncovalently bound to an antigenic molecule, in which the antigenic molecule is an exogenous antigen or
30 antigenic or immunogenic fragment or derivative thereof.

40. The kit of claim 39 which further comprises in a second container human antigen presenting cells.

35   41. A method of preventing cancer in an individual in whom such prevention is desired comprising the steps of:

(a)   sensitizing antigen presenting cells *in vitro*
       with a sensitizing- effective amount of a
       complex of a heat shock protein noncovalently
       bound to an antigenic molecule; and

5   (b)   administering to the individual a
       therapeutically effective amount of the
       sensitized antigen presenting cells.

42.   A method of preventing cancer in an individual in
10 whom such prevention is desired comprising administering to
the individual a therapeutically effective amount of
sensitized antigen presenting cells, in which the antigen
presenting cells have been sensitized *in vitro* with a complex
of a heat shock protein noncovalently bound to an antigenic
15 molecule.

43.   The method according to claim 3, 4, 6, 24, 25 or 42
in which the antigen presenting cells comprise macrophages.
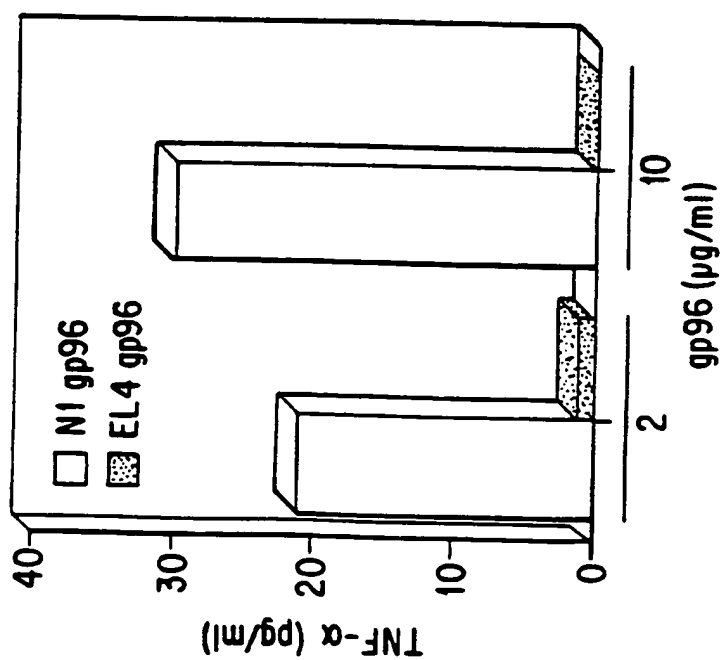
20

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FIG.1B

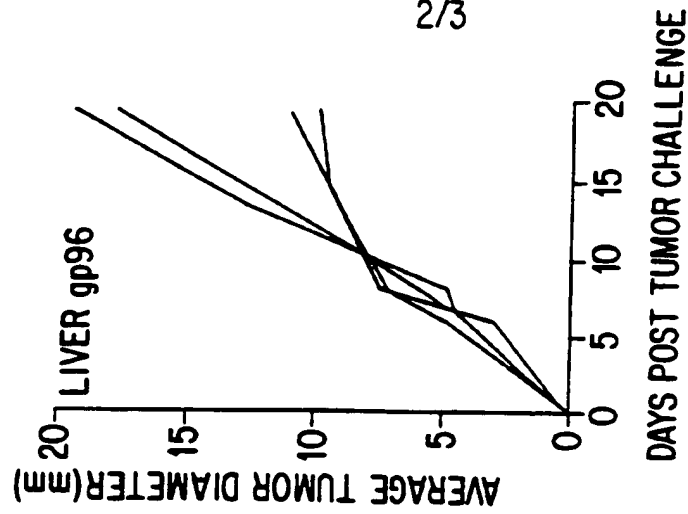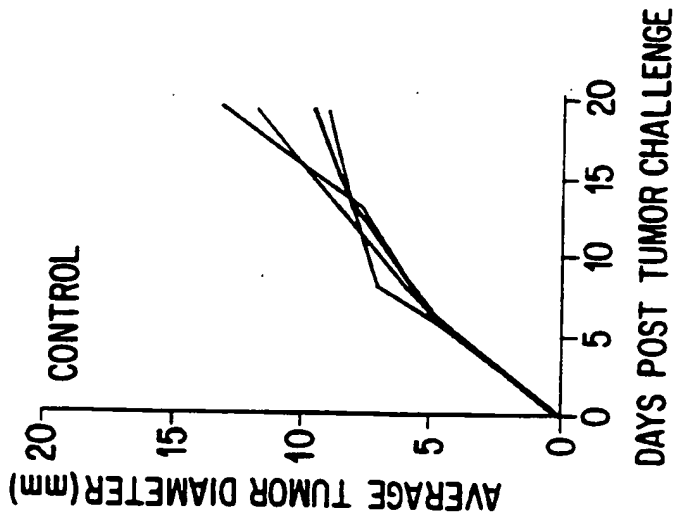FIG.1A

FIG. 2B

FIG. 2A

FIG. 1C

3/3
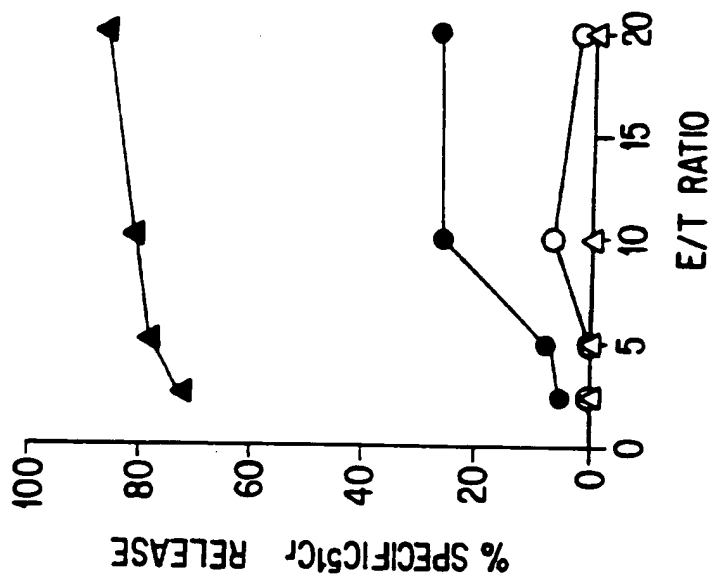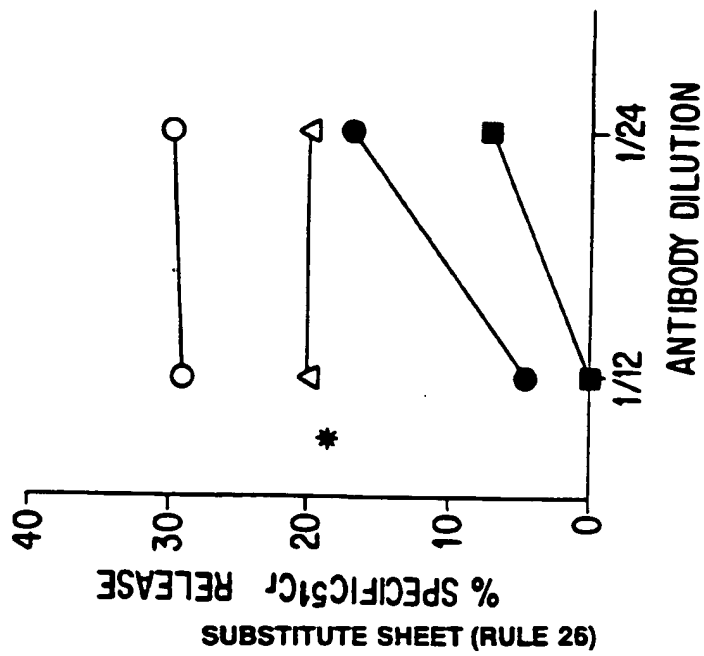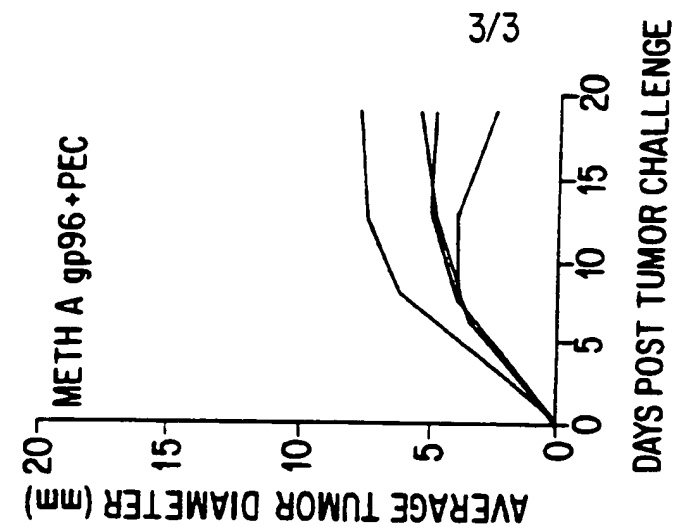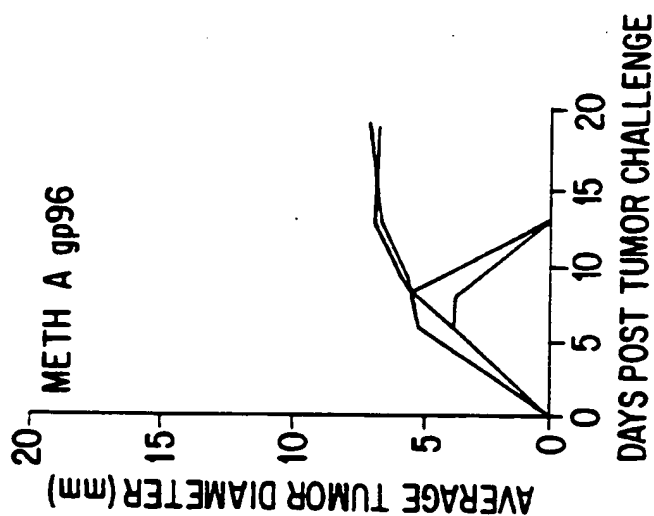

FIG. 2E

FIG. 2D

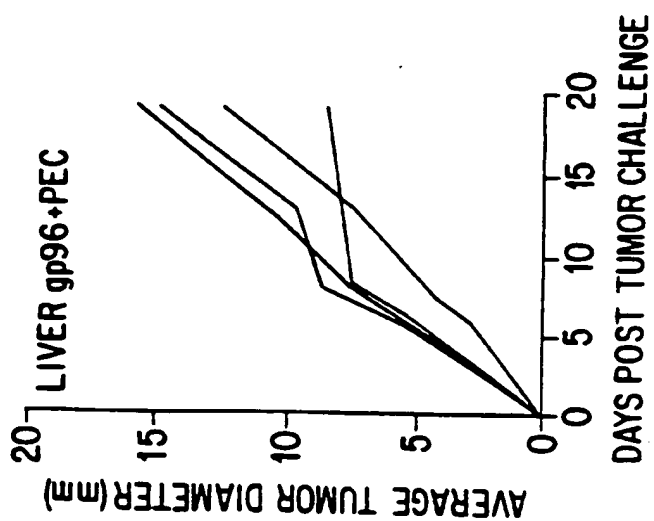FIG. 2C

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 39/385, 39/12, 39/02, 39/00, 35/12

US CL : 424/193.1, 204.1, 234.1, 274.1, 520, 573; 530/351

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/193.1, 204.1, 234.1, 274.1, 520, 573; 530/351

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SRIVASTAVA et al. Stress-induced proteins in immune response to cancer. Curr. Top. Microbiol. Immunol. June 1991, Vol.167, pages 109-123, especially pages 111-113 and 118-120.	1-21, 36-38, 41-43
X -- Y	GRABBE et al. Tumor antigen presentation by murine epidermal cells. J. Immunol. 15 May 1991, Vol.146, No. 10, pages 3656-3661, especially pp.3656-3666 and Figs. 1-3.	1-21, 36-38 ---------- 39-43
Y	SRIVASTAVA, P.K. Peptide-binding heat shock proteins in the endoplasmic reticulum: Role in immune response to cancer and in antigen presentation. Adv. Cancer Res. June 1993, Vol.62, pages 153-177, see entire document, especially Sections IV-VI.	1-21, 36-38, 41-43

| X | Further documents are listed in the continuation of Box C. | | See patent family annex. |

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 OCTOBER 1996	13 NOV 1996

| Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
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Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BLACHERE et al. Heat shock protein vaccines against cancer. J. Immunother. April 1993, Vol.14, pages 352-56, see entire document.	1-22, 36-43
Y	UDONO et al. Heat shock protein 70-associated peptides elicit specific cancer immunity. J. Exp. Med. October 1993, Vol.178, pages 1391-1396, see entire document.	1-22, 36-43
X — Y	WO 89/12455 A1 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 28 December 1989, see entire document.	22-35 ——— 1-21, 36-38, 41-43
Y	RAMSEY et al. Resolution of Chlamydial genital infection with antigen-specific T-lymphocyte lines. Infect. Immun. March 1991, Vol.59, No. 3, pages 925-931, see entire document.	22-35
Y	BARTHOLEYNS et al. Immune control of neoplasia by adoptive transfer of macrophages: Potentiality for antigen presentation and gene transfer. Anticancer Res. November 1994, Vol.14, No. 6B, pages 2673-2676, see entire document.	1-21, 36-43
Y	ULLRICH et al. A mouse tumor-specific transplantation antigen is a heat shock-related protein. Proc. Natl. Acad. Sci. USA. May 1986, Vol.83, pages 3121-3125, see entire document.	1-21, 36-43
Y	WO 94/02156 A1 (THE BOARD OF TRUSTEES OF LELAND STANFORD JUNIOR UNIVERSITY) 03 February 1994, see entire document.	1-21, 36-43
Y	CHOU et al. Adoptive immunotherapy of microscopic and advanced visceral metastases with in vitro sensitized lymphoid cells from mice bearing progressive tumors. J.Immunol. 01 September 1988, Vol.141, No. 5, pages 1775-1781, see entire document.	1-21, 36-43
Y	SUYU et al. Lymphocytes generated by in vivo priming and in vitro sensitization demonstrate therapeutic efficacy against a murine tumor that lacks apparent immunogenicity. J. Immunol. 15 July 1989, Vol.143, No. 2, pages 740-748, see entire document.	1-22, 36-43

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

USPAT, WPIDS, MEDLINE, BIOSIS, EMBASE
Search terms: antigen(w)(process? or presenting); (shock(2w)protein?) and (cancer or tumor?);
adoptive(10a)(immunother? or transfer) and (presenting) and (cancer or tumor? or infect?)

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-11, 14, 16-18, 19, and 36-43, drawn to methods of treating cancer with complexes of hsp70 and endogenous antigen, and a corresponding pharmaceutical composition or kit.

Group II, claims 1-10, 12, 14, 16-18, 19, and 36-43, drawn to methods of treating or preventing cancer with complexes of hsp90 and endogenous antigen and a corresponding pharmaceutical composition or kit.

Group III, claims 1-10, 13, 14, 16-18, 19, and 36-43, drawn to methods of treating or preventing cancer with complexes of gp96 and endogenous antigen and a corresponding pharmaceutical composition or kit.

Group IV, claims 1-11, 15, 16-18, 20-21, and 36-43, drawn to methods of treating or preventing cancer with complexes of hsp70 and exogenous antigen and a corresponding pharmaceutical composition or kit.

Group V, claims 1-10, 12, 15, 16-18, 20-21, and 36-43, drawn to methods of treating or preventing cancer with complexes of hsp90 and exogenous antigen and a corresponding pharmaceutical composition or kit.

Group VI, claims 1-10, 13, 15, 16-18, 20-21, and 36-43, drawn to methods of treating or preventing cancer with complexes of gp96 and exogenous antigen and a corresponding pharmaceutical composition or kit.

Group VII, claims 22-31, and 34-40, drawn to methods of treating infectious disease with complexes of hsp70 and antigen isolated from an infected autologous cell, and a corresponding pharmaceutical composition or kit.

Group VIII, claims 22-31, and 34-40, drawn to methods of treating infectious disease with complexes of hsp90 and antigen isolated from an infected autologous cell, and a corresponding pharmaceutical composition or kit.

Group IX, claims 22-31, and 34-40, drawn to methods of treating infectious disease with complexes of gp96 and antigen isolated from an infected autologous cell, and a corresponding pharmaceutical composition or kit.

Group X, claims 22-29, and 32-40, drawn to methods of treating infectious disease with complexes of hsp70 and exogenous antigen, and a corresponding pharmaceutical composition or kit.

Group XI, claims 22-29, and 32-40, drawn to methods of treating infectious disease with complexes of hsp90 and exogenous antigen, and a corresponding pharmaceutical composition or kit.

Group XII, claims 22-29, and 32-40, drawn to methods of treating infectious disease with complexes of gp96 and exogenous antigen, and a corresponding pharmaceutical composition or kit.

The inventions listed as Groups I-VI land VII-XII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical feature of Groups I-VI is the treatment of cancer, whereas the special technical feature of Groups VII-XII is the treatment of infectious disease. Immunotherapy of cancer is not deemed to be linked to immunotherapy of infectious disease. Therefore, the two special technical features make different contributions to the prior art associated with the respective diseases.

Further, the species hsp70, hsp90, and gp96, recited in claims 10, 35, and 38 do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: The species represent structurally and functionally distinct proteins/protein families (hsp70, hsp90, and gp96) which are not deemed capable of functioning identically with different antigens, antigen-presenting cells, and diseases. Therefore, the species represent un-linked contributions to the prior art.

And finally, the use of endogenous antigens in the complex (claims 19 and 30-31) versus exogenous antigens in the complex (claims 20-21 and 32-33) does not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: The special technical feature of endogenous antigens is that they co-purify with the individual's heat shock protein and need not be further purified or characterized, whereas the special technical feature of exogenous antigens is that they are combined with a purified heat shock protein from which the endogenous antigen(s) has/have been removed. Thus, the heat shock protein (product by process) which is complexed with endogenous antigens and used in a method of treatment is distinct from and not linked to the heat shock protein (product by process) which is complexed with exogenous antigens, and the different products represent distinct contributions to the prior art.